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Preservation of *Neisseria gonorrhoeae* at −20°C

PIERRE S. HARBEC* AND PIERRE TURCOTTE

Laboratoire de Santé Publique du Québec, Sainte-Anne-de-Bellevue, Québec, Canada H9X 3R5

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To explore the feasibility of preserving *Neisseria gonorrhoeae* at −20°C, we studied its viability quantitatively and qualitatively for 12 and 18 months, respectively, in the following media: a gelatin-based medium used mainly to prepare dried gelatin discs (S. Yamai, Y. Obara, T. Nikkawa, Y. Shimoda, and Y. Miyamoto, Br. J. Vener. Dis. 55:90–93, 1979), a simplified version (LSPQ preservation medium), and Trypticase soy broth with 10% (vol/vol) glycerol, a medium commonly used for preservation at −70°C. The latter was studied for 4 months only. Four reference strains and two clinical isolates of *N. gonorrhoeae* were used. The storage temperature was rigorously preadjusted and monitored at −20 ± 1°C during the entire project. After 12 months of storage, all strains remained viable in both gelatin-based media, whereas a significant loss of viability was observed in Trypticase soy broth–10% glycerol after only 4 months. After 18 months, five strains were still viable in both gelatin-based media and no significant difference was observed between antimicrobial susceptibility results and those of the original strains preserved at −70°C. On the basis of these results, we believe that LSPQ preservation medium represents a good alternative for the storage of *N. gonorrhoeae* at −20°C for at least a year. Furthermore, it is easy to prepare and use and can be stored at 4 to 8°C for a year prior to use.

In most laboratories, bacterial strains need to be maintained for quality control, teaching, and research. To do this, workers must have the facilities to preserve these microorganisms. Unfortunately, many laboratories have access only to a −20°C freezer rather than the recommended −60°C (or lower) unit. Consequently, when Trypticase soy broth or a similar medium with 10 to 15% glycerol is used for preservation at −20°C, certain fastidious microorganisms, such as *Neisseria gonorrhoeae*, *Haemophilus* spp., and *Campylobacter* spp., may survive for no more than a few months.

Having received reports of this problem from many hospital laboratories throughout the province of Québec, Canada, we undertook to study the feasibility of preserving a delicate microorganism such as *N. gonorrhoeae* at −20°C for a longer period of time.

Some researchers (4, 5, 7, 13, 14) have worked with bacteria preserved −20°C, but only two groups (10, 15) have done such a study with *N. gonorrhoeae*. In 1979, Yamai et al. (15) described a medium used for the preparation of dried gelatin discs as a means of preservation. They also observed that *N. gonorrhoeae*, when suspended in this liquid medium and kept frozen at −20°C, remained viable for up to 6 months. On the basis of this observation, our objective was to verify the efficacy of their medium while simplifying its preparation and use.

MATERIALS AND METHODS

Strains. Six strains of *N. gonorrhoeae* were used: ATCC 31426, a β-lactamase-positive strain; ATCC 43069, a strain recommended by the National Committee for Clinical Laboratory Standards for quality control of media; ATCC 49226, the National Committee for Clinical Laboratory Standards reference strain for antimicrobial susceptibility testing; ATCC 35201, an arginine-, hypoxanthine-, and uracil-requiring (AHU^-) strain; LSPQ 3381, a penicillin- and tetracycline-resistant strain; and LSPQ 3626, a proline-, citrulline-, and uracil-requiring (PCU^-) strain. The latter are two clinical isolates deposited in our culture collection.

* Corresponding author. Mailing address: Laboratoire de Santé Publique du Québec, 20045, chemin Sainte-Marie, Sainte-Anne-de-Bellevue (Québec), Canada H9X 3R5. Phone: (514) 457-2070. Fax: (514) 457-6346.

Preservation media. Yamai and LSPQ preservation media were compared to Trypticase soy broth (Difco Laboratories, Detroit, Mich.) with 10% (vol/vol) glycerol (TSBG), a medium commonly used to preserve bacteria at −70°C (2, 9).

Yamai medium was prepared as described previously (15). It consists of reagents A (5% glucose [Difco], 3% skim milk [Difco], and 0.6% activated charcoal [Norit SG, C-5510; Sigma Chemical Co., St. Louis, Mo.] in deionized water sterilized at 110°C for 10 min), B (5% Na–L-ascorbate [Sigma] in deionized water sterilized by filtration and kept frozen at −20°C prior to use); and C (20% solution of gelatin [Difco] in deionized water sterilized at 121°C for 20 min). Reagents A and C were kept refrigerated (4 to 8°C) prior to use. All of the reagents can be kept for a long time at the temperatures mentioned above. For preparation of the medium, solutions A, B, and C are combined aseptically in a ratio of 1:0.2:1, respectively, and used immediately. For preparation of LSPQ preservation medium, 5 g of glucose (Difco), 0.6 g of activated charcoal (Sigma), 3 g of skim milk (Difco), and 10 g of gelatin (Difco) were dissolved in 100 ml of deionized water and autoclaved at 110°C for 10 min. After sterilization, this solution was distributed in 3.3-ml volumes in screw-cap tubes (13 by 100 mm). Na–L-ascorbate was omitted from this medium because its protective action is effective only when the medium is air dried (13). After preparation, the medium was stored at 4 to 8°C prior to use.

Storage temperature. To control temperature during this study, we used a vertical freezer (Foster QH-2-T; Foster Refrigeration of Canada Ltd., Drummondville, Québec, Canada) preadjusted to −20 ± 1°C with two type T thermocouples, each one inserted into cryotubes filled with preservation medium and placed, one in the front and the other in the back of storage boxes, inside the freezer. A thermocouple thermometer (Barnant 100, model 600-2820; J-K-T, Barnant Co., Barrington, Ill.) was used to monitor the temperature daily. It is important to point out that this is not a frost-free unit. Such units are not recommended for laboratory storage (1) because the frost-free condition is achieved by alternate warming and cooling cycles, which can result in further damage to cells and a concomitant loss of viability (3).

Preparation of bacterial suspensions. All strains were subcultured on chocolate agar from a 0.1-ml inoculum of a bacterial suspension in Mueller-Hinton broth diluted from a 0.5 McFarland standard to obtain approximately 10^4 CFU/ml; the cultures were incubated for 16 to 20 h at 35°C in a 5% CO_2, humid atmosphere. For each strain, the suspensions for Yamai medium were prepared in the following manner. Reagents A, B, and C, preheated at 35°C up to the liquefaction of gelatin, were combined for a 33-ml final volume and kept at 35°C in a water bath prior to use. For the LSPQ preservation medium, eight tubes containing 3.3 ml of medium were placed in a water bath at 35°C to liquefy the medium. For both media, a volume of 3.3 ml was poured onto a young pure culture of *N. gonorrhoeae* on chocolate agar just taken out of the incubator. The use of a young culture is important to reduce, as much as possible, the formation of a gummy inoculum (8); the warm agar plate prevents solidification of the suspension medium. Immediately, the bacterial suspension was prepared by scraping the surface of the agar with a spreader. The dense and thick bacterial suspension was collected with a Pasteur pipette, deposited in a 30-ml tube, and kept at 35°C in a water bath. For each strain and each medium, eight chocolate

agar plates were used to prepare approximately 20 ml (final volume) pooled bacterial suspensions.

Subsequently, the suspensions were vigorously agitated on a mechanical vortex mixer and aliquoted as 0.5 ml per vial in 27 cryovials (no. 3-40711; Nunc, Roskilde, Denmark). Three additional samples of 1.5 ml for each strain and each medium were prepared for qualitative analysis. As soon as filled, each batch of samples was rapidly placed in its respective freezing box at −20°C. The same procedure was used to prepare 15 samples of each strain in TSBG, except that no additional vials were prepared for qualitative analysis.

Viability determination (CFU per milliliter). The viability of *N. gonorrhoeae* was determined quantitatively by plate counts on chocolate agar. The frequency of sampling was as follows: once before freezing, a week after, and subsequently every 2 months for up to 12 months. These determinations were made, each time, on three samples for each strain and for both gelatin-based preservation media. Briefly, counts were performed in the following manner. Three frozen cryovials of each strain were placed in a water bath at 35°C for 15 min. The content of each cryovial (0.5 ml) was first diluted with approximately 1 ml of prewarmed (35°C) Mueller-Hinton broth, pipetted from the first tube (9.5 ml), and vigorously agitated on a mechanical vortex mixer after each dilution; once diluted, the content of the cryovial was transferred to the latter tube of Mueller-Hinton broth. Five serial 10-fold dilutions in Mueller-Hinton broth were carried out. A 0.1-ml aliquot of each of the last three dilutions was plated on chocolate agar. The plates were examined and counted after 48 h of incubation. The oxidase test and the Gram stain were used to confirm the presence of *N. gonorrhoeae*. For samples frozen in TSBG, the same procedure was used except that the frequency of sampling was once before freezing, a week after, and subsequently at 1, 2, and 4 months.

Qualitative analysis of viability was done with three cryotubes (1.5 ml each) for both gelatin-based media and for each strain in the following manner. With a rigid, homemade microspatula, a frozen piece of approximately 50 μl of gelatin-based medium was removed from a cryotube and inoculated onto chocolate agar prewarmed at 35°C for 30 min. The cryotube was immediately replaced at −20°C. The melted gelatin was then spread with a loop on the first half of the petri dish and streaked on the second half to obtain distinct colonies. The plates were incubated for 48 h as described above. The growth obtained in the first half of the petri dish was interpreted as follows: 4+, confluent; 3+, mixture of confluent and isolated colonies; 2+, ≥100 colonies; 1+, <100 colonies. The frequency of this determination was the same as for the quantitative determination, but the sequential samples were always picked up from the same cryotube. Also, after 18 months, a final qualitative viability test was performed on these three remaining cryovials for each strain and medium. Their whole content was liquefied in a water bath at 35°C for 15 min, and with a Pasteur pipette, 6 drops of each suspension were placed on chocolate agar and processed as mentioned previously.

Stability of the strains. Quantitative antimicrobial susceptibility testing results were generated in accordance with the National Committee for Clinical Laboratory Standards protocol (9) against penicillin, tetracycline, spectinomycin, ceftriaxone, ciprofloxacin, and cefixime. Comparison of MICs for strains preserved at −70°C was used to confirm the stability of the strains (6) after storage at −20°C.

Quantitative analysis. (i) Survival curve. To simplify strain comparisons, the survival curves were standardized. They were obtained by plotting, for each time interval, the mean value of the viable units recovered in the three samples, divided by the mean initial viability count, multiplied by a hundred.

(ii) Viability loss. The overall viability loss observed over the study period was expressed as the log of the difference between the initial number of viable units before freezing and those counted after 4 or 12 months of storage.

Qualitative analysis. Each strain was studied in triplicate; the final result is reported as an average growth score.

RESULTS

After 12 months of storage at −20 ± 1°C, all strains remained viable in the LSPQ and Yamai preservation media. In TSBG, a high loss of viability was observed (Table 1) for most of the strains after only 4 months of storage (Fig. 1).

By qualitative analysis, viability ranged from 4+ to 2+ in all cryotubes and for all strains, up to 12 months. With these same samples, after 18 months, five strains remained viable, although the amount of bacterial growth differed from strain to strain by a few colonies to confluent growth; only ATCC 49226 was nonviable in both media.

The antimicrobial susceptibility testing results for these five strains after their long storage showed no significant difference (MIC ± 1 dilution) compared with the MICs for the original strains preserved at −70°C in TSBG (data not shown).

TABLE 1. Logarithmic loss of viability of six strains of *N. gonorrhoeae* after storage at −20°C

Strain	Medium	Viability		
		Before freezing	After 12 months	Loss
ATCC 31426	LSPQ	9.14	7.66	1.48
	Yamai	9.30	7.14	2.16
	TSBG	9.53	7.61[a]	1.92[a]
ATCC 43069	LSPQ	9.98	8.17	1.81
	Yamai	10.04	8.30	1.74
	TSBG	9.97	6.71[a]	3.26[a]
ATCC 49226	LSPQ	9.20	5.85	3.35
	Yamai	9.30	3.91	5.39
	TSBG	9.80	5.64[a]	4.16[a]
ATCC 35201	LSPQ	9.04	6.23	2.81
	Yamai	9.04	5.50	3.54
	TSBG	8.86	4.04[a]	4.82[a]
LSPQ 3381	LSPQ	9.55	6.98	2.57
	Yamai	9.79	6.86	2.93
	TSBG	9.71	7.85[a]	1.86[a]
LSPQ 3626	LSPQ	8.73	6.11	2.62
	Yamai	8.84	4.00	4.84
	TSBG	9.14	5.84[a]	3.30[a]

[a] Viability after only 4 months of storage.

DISCUSSION

Preservation of strains is important for all laboratories, but for small laboratories, many difficulties arise with some fastidious bacteria because the only facility generally available for preservation is a −20°C freezer. Our objective was to provide these laboratories with a reliable method of preservation. *N. gonorrhoeae* was chosen as the challenge microorganism in our study because one of us, as curator of a culture collection in a public health laboratory, observed that many small hospital laboratories were experiencing great difficulties preserving this species for their quality control programs. To date, the gelatin-based medium of Yamai (15) was the only one reported to be capable of preserving this microorganism at −20°C for up to 6 months.

For our preliminary study, strains ATCC 31426, ATCC 43069, and ATCC 49226 were chosen because of their popularity as quality control strains in our province; LSPQ 3381 was chosen because of its resistance to antibiotics; and ATCC 35201 and LSPQ 3626 were chosen for their slow growth and fragile viability. The number of strains used was limited to six because of the numerous samplings needed to track closely the survival of each strain throughout the project.

We expected strains ATCC 35201 and LSPQ 3626 to be the most vulnerable and difficult to preserve. Unexpectedly, ATCC 49226 was the only strain that did not survive for up to 18 months. This suggests its greater vulnerability to adverse environmental conditions (temperature and medium) and leads us to recommend the inclusion of this reference strain in similar future studies.

The number of samples used for the quantitative analyses was planned for a period of 12 months only because, according to Yamai et al. (15) and Obara et al. (10), we could not expect viability for a period exceeding 6 months. Our success in preserving all of the strains for up to 12 months in both media is probably due to the meticulous preparation of the young bacterial suspensions, the volume contained in each cryotube (0.5 ml compared with the 0.1 ml used by the above-mentioned investigators), and, possibly, the rigorous monitoring and maintenance of the temperature at −20 ± 1°C during the

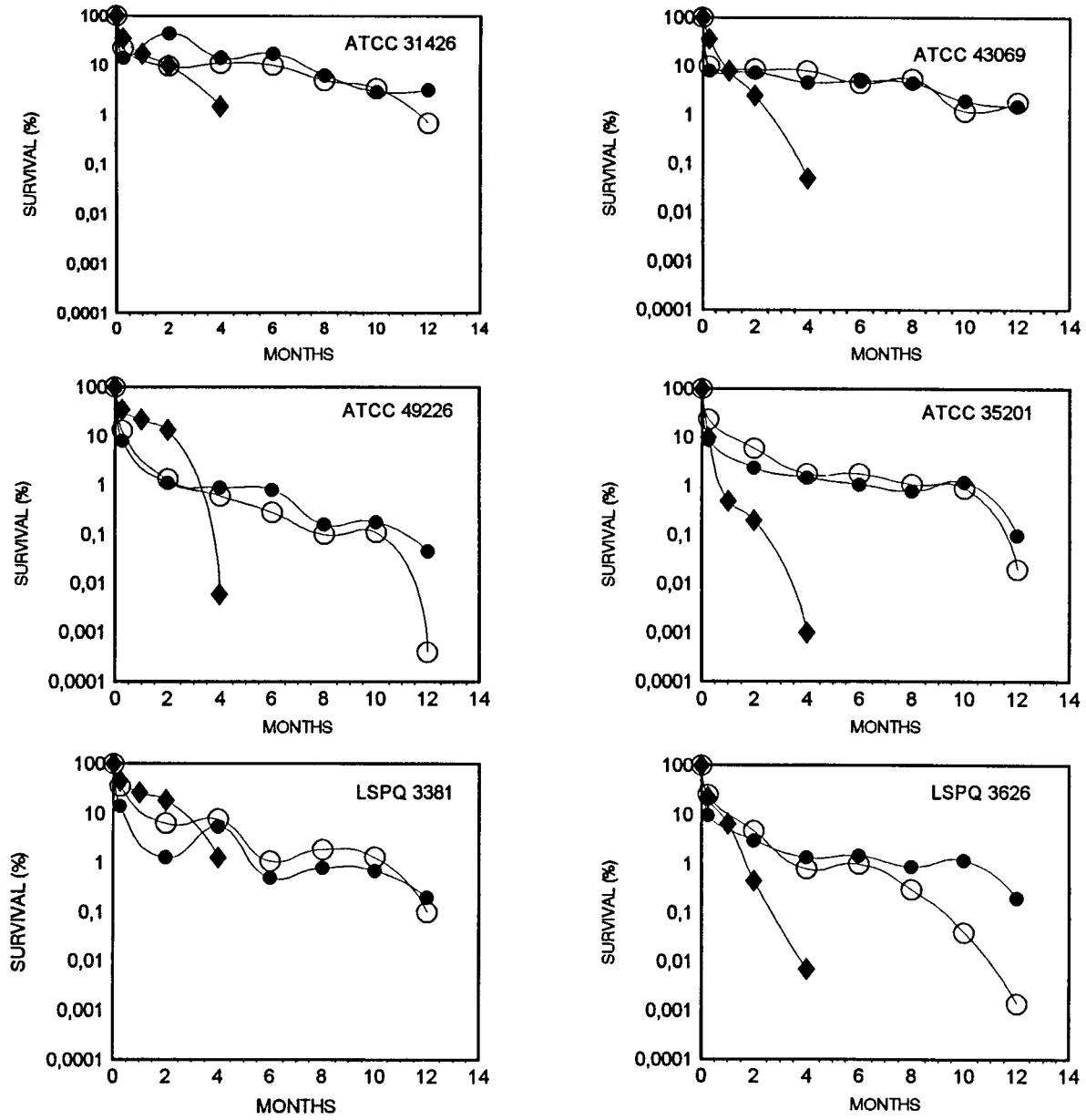

FIG. 1. Percentage of survival of six strains of *N. gonorrhoeae* preserved at −20°C in three different media. Symbols: ●, LSPQ preservation medium; ○, Yamai preservation medium; ◆, TSBG.

course of the project to obtain reliable data at this critical temperature. With this in mind, users of this method must be aware that temperatures only a few degrees higher or lower than −20°C may shorten or increase the storage life of the bacteria. The qualitative analyses were conducted to observe the prolonged survival of a bacterial suspension subjected to multiple samplings without thawing. This sampling method (11) was used in our laboratory for 5 years on most of our strains stored at −70°C in TSBG. Our results demonstrate that it is possible to recover the bacteria but the procedure used is troublesome if the hard medium is not allowed to soften at room temperature. Our experience has been that the gelatin residues are very difficult to remove from the microspatula used for this purpose, and when samples are flamed, aerosols are created. In view of these facts, we do not recommend this method of repeated sampling.

The experiment with TSBG was planned for a period of 4 months only because the information obtained from a few hospitals in our province stated that *N. gonorrhoeae* would not survive for more than 6 weeks to 2 months at −20°C. In a similar context, Knapp and Rice (6) indicated that some isolates preserved at −20°C in Trypticase soy broth with 20% glycerol may survive storage for up to 2 weeks. Our study allowed us to observe viability for up to 4 months for all of the strains. However, we noted a sharp drop in bacterial counts at the end of this period.

The antimicrobial susceptibility testing performed on the five viable strains confirmed the stability of their susceptibility pattern (6) in both gelatin-based preservation media after 18 months of storage.

In conclusion, we believe that LSPQ preservation medium represents a good alternative for the storage of strains of

N. gonorrhoeae at −20°C for at least a year. The advantages over Yamai medium are simple preparation and use; moreover, prior to use, the complete medium can be stored for up to a year in tightly sealed screw-cap tubes at 4 to 8°C (12). Furthermore, a recent qualitative assay with *Campylobacter jejuni* ATCC 33291 in our medium and under the same conditions has shown maintenance of viability of the strain after 12 months of storage. This medium may thus be useful for the preservation of a variety of fastidious strains for at least a year.

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